Supporting Information

A Viscosity-Responsive Mitochondria-Targeting Probe for Rapid Imaging of Fatty Liver Disease

Jiamin Liu,[#a] Hui Zhou,[#*a] Deyi Li,[b] Haotong Yin,[a] Yi Zhou,[a] Yuquan Ji,[a] Yujing Zhang,[a] Xinyue Zhang,[a] Ben Wang,[a] Chao Yin *[a] and Quli Fan [a]

[a] State Key Laboratory of Flexible Electronics (LoFE), Institute of Advanced Materials (IAM), Jiangsu Key Laboratory of Smart Biomaterials and Theranostic Technology, Jiangsu National Synergetic Innovation Center for Advanced Materials (SICAM), School of Materials Science and Engineering, Nanjing University of Posts and Telecommunications, Nanjing, 210023, China.
E-mail: iamhzhou@njupt.edu.cn; iamcyin@njupt.edu.cn
[b] Fujian Haixi Pharmaceuticals Co., Ltd, Fuzhou, 350028, China.

# J. Liu and H. Zhou contributed equally to this work.

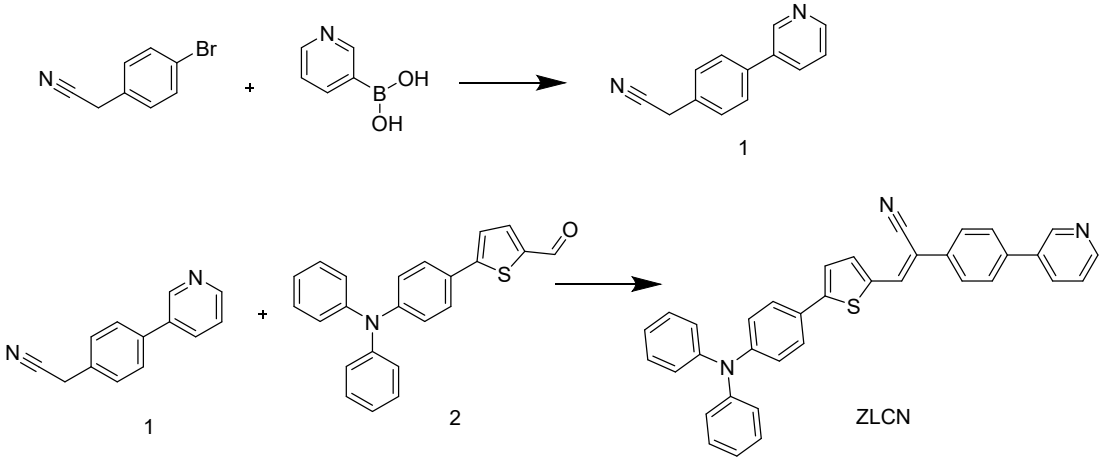
1. Experimental procedures

1.1 Materials and instrumentation

4-Bromophenylacetonitrile, Pyridin-3-ylboronic acid, 5-(4-(diphenylamino)phenyl)thiophene-2-carbaldehyde were purchased from Bide Pharmatech Co.,Ltd. MitoTracker Green were purchased from Betotime Biotechnology. Fetal bovine serum (FBS) was obtained from Thermo Fisher Scientific (USA). All other reagents and solvents were used directly from the corresponding supplier without furtherpurification. Nystatin was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. All starting materials were purchased from Sigma, Energy, Aladdin and use directly.

^{1}H-nuclear magnetic resonance (NMR) and ^{13}C-NMR spectra of all compounds were acquired using a Bruker Avance III 400 spectrometer. Absorption data was measured by Shimadzu UV-3600 ultraviolet-visible-near-infrared (UV-Vis-NIR) spectrophotometer. Fluorescence spectra were measured on a Fluorolog-3 spectrofluorometer (Horiba Jobin Yvon, France). The 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay was performed by a BioTek Synergy HTX microplate reader. Confocal fluorescence imaging was conducted on ZEISS LSM880 laser scanning confocal microscope. In vivo imaging was performed by the IVIS LUMINA K Series lll.

1.2 Synthetic routes of desired molecules



Synthesis of 1

4-Bromophenylacetonitrile (196 mg, 1 mmol) and an excess of pyridin-3-ylboronic acid (148 mg, 1.2 mmol) were added to a 50 mL reaction tube. $Pd(PPh_3)_4$ (46 mg, 0.04 mmol) was then introduced as the catalyst, followed by anhydrous potassium carbonate (415 mg, 3 mmol) as the base. A solvent mixture consisting of 1,4-dioxane (8 mL) and deionized water (2 mL) was subsequently added. The reaction was carried out under a nitrogen atmosphere at 80°C in an oil bath with continuous stirring for 12 hours. After completion, the reaction mixture was allowed to cool to room temperature and extracted three times with dichloromethane (DCM). The combined organic layers were dried over

anhydrous sodium sulfate for 30 minutes and then filtered under reduced pressure. The crude product was purified by silica gel column chromatography using petroleum ether/ethyl acetate (1:1, v/v) as the eluent, affording the target compound as a white crystalline solid (161 mg, 83% yield), which was used directly in the subsequent step without further purification.^{1}H NMR (400 MHz, DMSO) δ 8.91 (s, 1H), 8.58 (d, *J* = 5.6 Hz, 1H), 8.07 (dt, *J* = 7.9, 2.0 Hz, 1H), 7.76 (d, *J* = 8.4 Hz, 2H), 7.48 (d, *J* = 8.4 Hz, 3H), 4.11 (s, 2H).

Synthesis of ZLCN

Compound 1 (155 mg, 0.8 mmol), compound 2 (319 mg, 0.9 mmol), and potassium hydroxide (73 mg, 1.30 mmol) were dissolved in anhydrous ethanol (5 mL). The reaction mixture was stirred at room temperature for 12 hours until completion. After the reaction, the crude product was purified by silica gel column chromatography using petroleum ether/ethyl acetate (5:1, v/v) as the eluent, affording compound 3 as an orange crystalline solid (267 mg, 63% yield). 1H NMR (400 MHz, DMSO) δ 8.98 (dd, *J* = 8.0, 3.1 Hz, 1H), 8.60 (dd, *J* = 4.8, 1.6 Hz, 1H), 8.22 – 8.10 (m, 1H), 7.94 – 7.82 (m, 3H), 7.82 – 7.43 (m, 5H), 7.38 – 7.26 (m, 6H), 7.14 – 7.04 (m, 3H), 6.93 (dd, *J* = 57.2, 8.1 Hz, 5H).

1.3 AIE performance test

A 2 mM stock solution of compound 3 was first prepared in dimethyl sulfoxide (DMSO). Aliquots of this stock solution were then transferred into multiple EP tubes, followed by the addition of deionized water and DMSO to achieve a final concentration of 10 μM while varying the water content to 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, and 80% (v/v). After thorough mixing, the fluorescence emission spectra of the resulting solutions were immediately recorded.

1.4 Fluorescence emission measurement in ethanol/glycerol mixtures

Ten gradient ethanol/glycerol solvent systems were prepared, with the glycerol fraction gradually increasing from 0% to 90% (v/v). An equal volume of ZLCN stock solution was then added to each system to achieve a final concentration of 10 μM. The fluorescence emission spectra were recorded in the range of 500–700 nm under 490 nm excitation to investigate the spectral response of ZLCN to solvent viscosity variations.

1.5 Preparation of reactive ions and molecules

Sodium ion (Na^+) was prepared by dissolving sodium sulfate (Na_2SO_4) in deionized water. Copper (II) ion (Cu^{2+}) was obtained by adding copper (II) oxide (CuO) to dilute sulfuric acid (H_2SO_4) under stirring until complete dissolution, resulting in copper (II) sulfate solution. Iron (II) ion (Fe^{2+}) was generated by dissolving iron powder in dilute sulfuric acid, forming iron (II) sulfate solution. Nitrite ion (NO_2^-) was prepared by dissolving sodium nitrite ($NaNO_2$) in deionized water, while sulfite ion (SO_3^{2-}) was obtained by dissolving sodium sulfite (Na_2SO_3) in water. Cysteine (Cys) was dissolved in deionized water to the desired concentration. Superoxide anion (O_2^-) was generated by dissolving potassium superoxide (KO_2) in anhydrous dimethyl sulfoxide (DMSO) with ultrasonic agitation. To prepare peroxynitrite ($ONOO^-$), a mixture of hydrochloric acid (HCl) and hydrogen peroxide (H_2O_2) solution was combined with a mixture of sodium nitrite ($NaNO_2$) and sodium hydroxide (NaOH)

solutions, and the resulting mixture was poured into a small amount of ice, manganese dioxide (MnO_2) solid was then added to remove excess hydrogen peroxide (H_2O_2). Hydrogen peroxide (H_2O_2) was prepared by diluting 30% H_2O_2 with deionized water to the required concentration. Ammonium ion (NH_4^+) was obtained by dissolving ammonium chloride (NH_4Cl) in deionized water.

1.6 Cellular experiments

Cell culture: The AML12, 3T3, 4T1, and Hep1-6 cell lines were gifted from Nanjing University. These cells were cultured in DMEM with high glucose, supplemented with 10% fetal bovine serum and 1% antibiotics (penicillin/streptomycin; 100 U/mL), at 37°C in a 5% CO_2 atmosphere.

Cell Viability: The cytotoxicity of ZLCN was evaluated using the standard methyl thiazolyl tetrazolium (MTT) assay in AML12 cells. Briefly, the cells were seeded in 96-well plates at a density of 6000 cells per well and cultured for 24 h. The cells were then treated with various concentrations of ZLCN (0, 5, 10, 20, 40, 80, 160 μM) for an additional 24 h. After treatment, the medium was discarded, and the cells were washed twice with PBS. Fresh medium containing 0.5 mg/mL MTT solution was added, and the cells were incubated for another 4 h. The medium was then removed, and the cells were washed three times with PBS. Finally, 150 μL DMSO was added to dissolve the precipitated formazan crystals. Absorbance was measured using a microplate reader at 490 nm.

Colocalization experiments of mitochondria: AML12 cells were incubated with ZLCN solution for 6 h, followed by PBS washing to remove any unbound dye. The cells were then stained with MitoTracker Green under light-protected conditions for 30 min, followed by two additional PBS washes. Fluorescence imaging was performed using confocal laser scanning microscopy (CLSM). ZLCN was excited at 488 nm, and its emission was collected in the 580–650 nm range. MitoTracker Green was also excited at 488 nm, with its emission detected in the 500–530 nm range. Channel crosstalk calibration was conducted to ensure no nonspecific fluorescence interference within the selected spectral ranges.

Viscosity responsiveness experiment in vitro: AML12 cells were seeded in confocal culture dishes and incubated until they reached 40–50% confluence. The cells were divided into a control group and a drug-treated group. 1) Control group: Cells were incubated with 60 μM ZLCN in DMEM for 6 h. 2) Drug-treated group: Cells were pretreated with 25 μM nystatin for 20 min to enhance membrane permeability, followed by washing twice with PBS. Then, cells were incubated with 60 μM ZLCN in DMEM for another 6 h. After incubation, both groups were washed three times with PBS to remove any residual fluorescence probe. Fluorescence imaging was performed using confocal laser scanning microscopy (CLSM) (Ex/Em = 488 nm/610 nm).

1.7 In vivo imaging of mice

All the mice experiments were conducted in accordance with the guidelines of the Laboratory Animal Center of Jiangsu KeyGEN Biotech Corp., Ltd and approved by the Animal Ethics Committee of Simcere BioTech Corp., Ltd. To monitor in vivo liver viscosity in a mouse model of

fatty liver disease, Balb/c mice were randomly divided into two groups: a control group and an experimental group. The experimental group was fed a high-fat diet (60 kcal% fat) for 8 weeks, while the control group received a normal diet. Both groups were intravenously injected with ZLCN (100 μL, 150 μM) for in vivo fluorescence imaging. Prior to imaging, the Balb/c mice were fasted for 12 hours to minimize potential interference from dietary fluorescence. After 60 minutes post intravenous injection, the mice were anesthetized and sacrificed, and major organs were isolated for biodistribution analysis.

2. Supplementary Figures

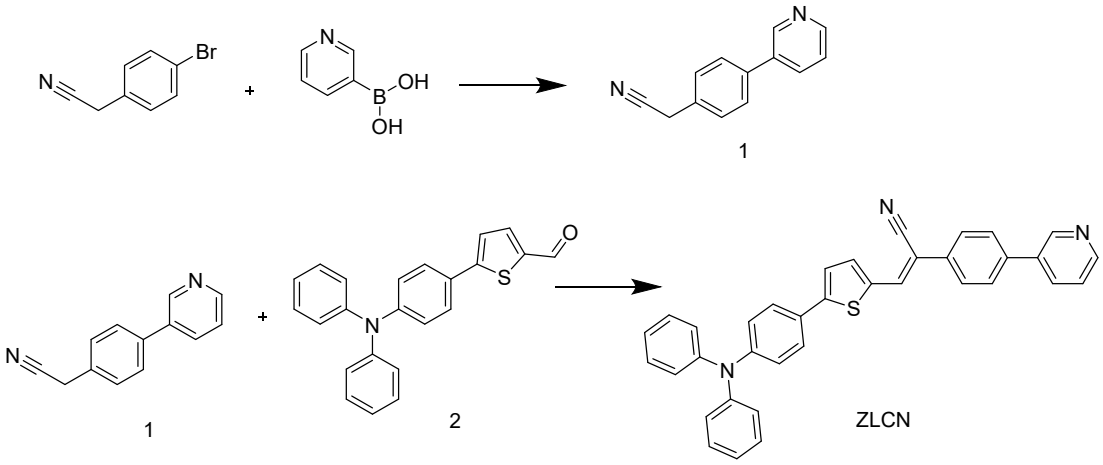


Fig. S1. Synthetic route of the probe ZLCN

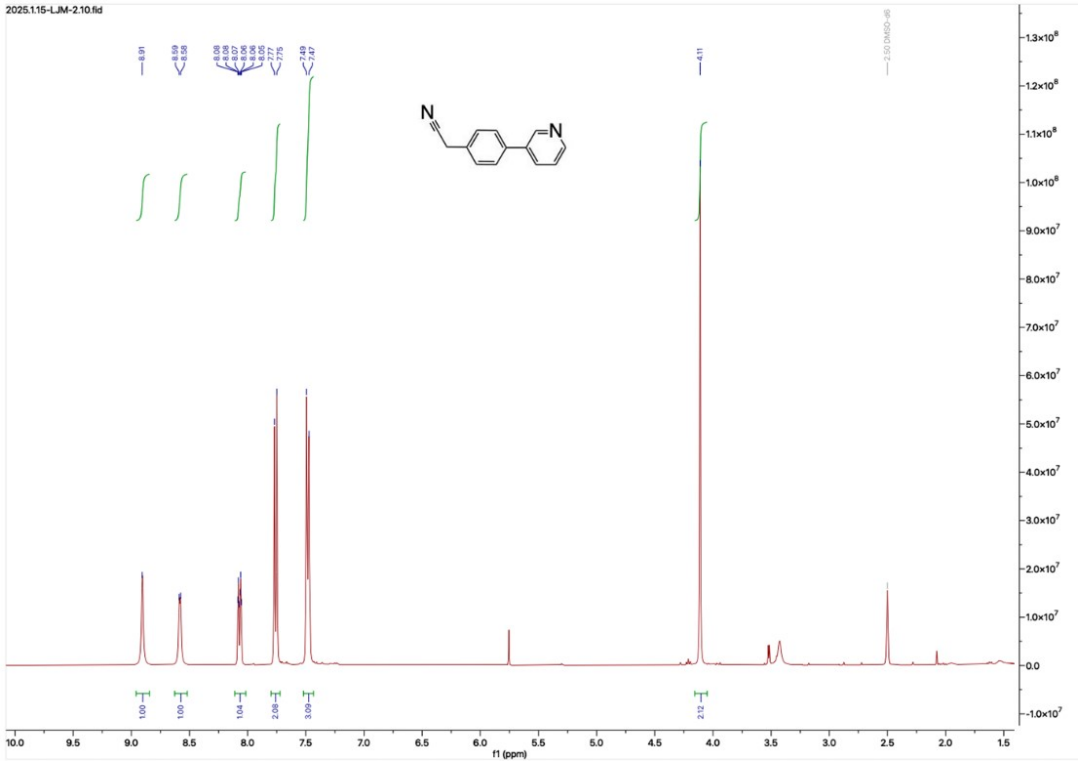


Fig. S2. ^{1}H NMR spectra of compound **1** in DMSO-d_6.

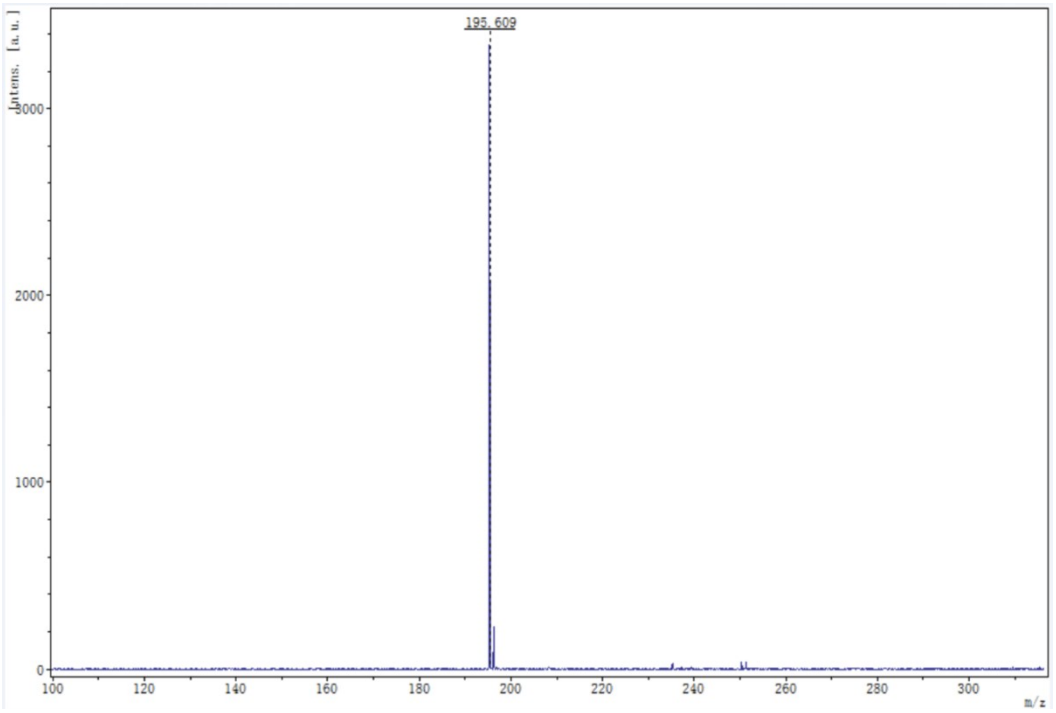


Fig. S3. Maldi-Tof of compound 1.

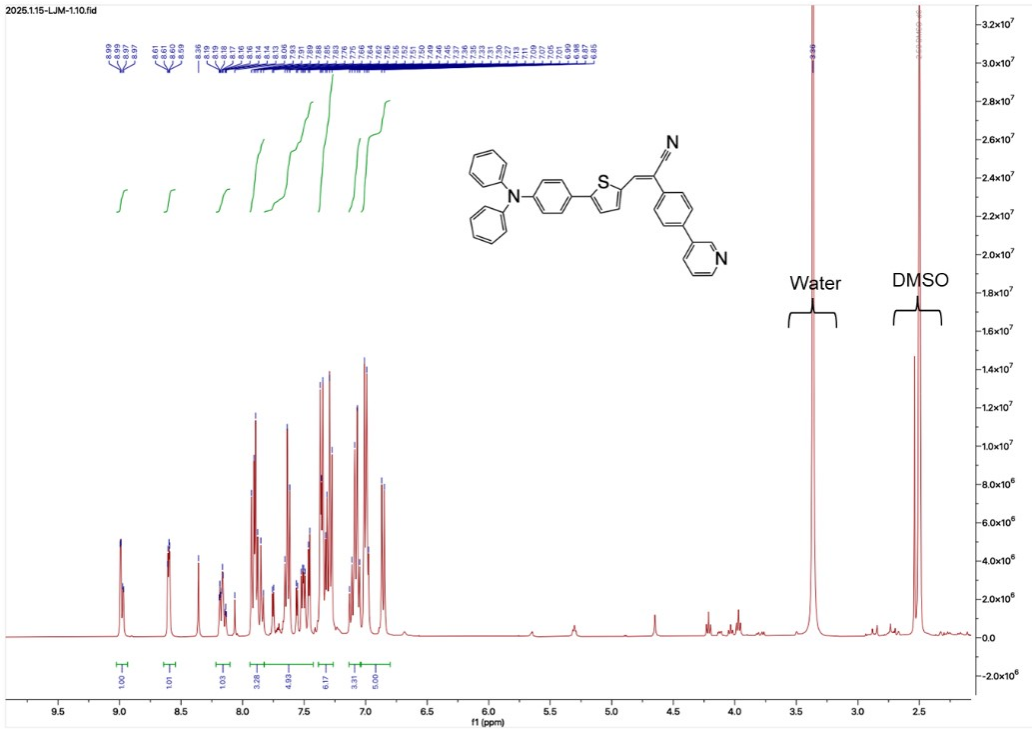


Fig. S4. ^{1}H NMR spectra of compound **ZLCN** in DMSO-d_6.

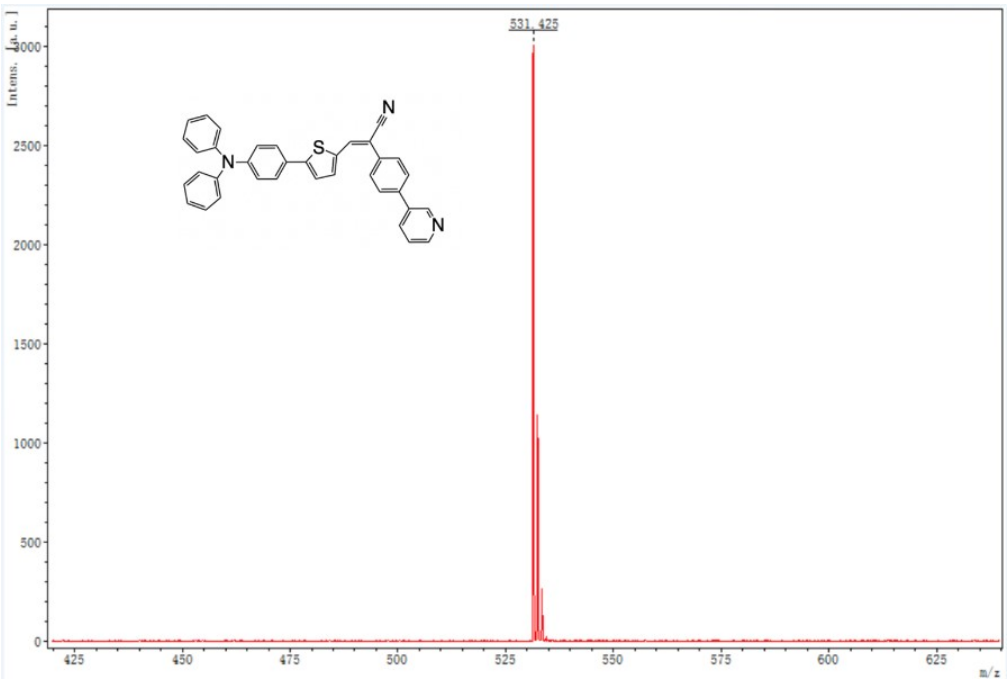


Fig. S5. Maldi-Tof of compound ZLCN.

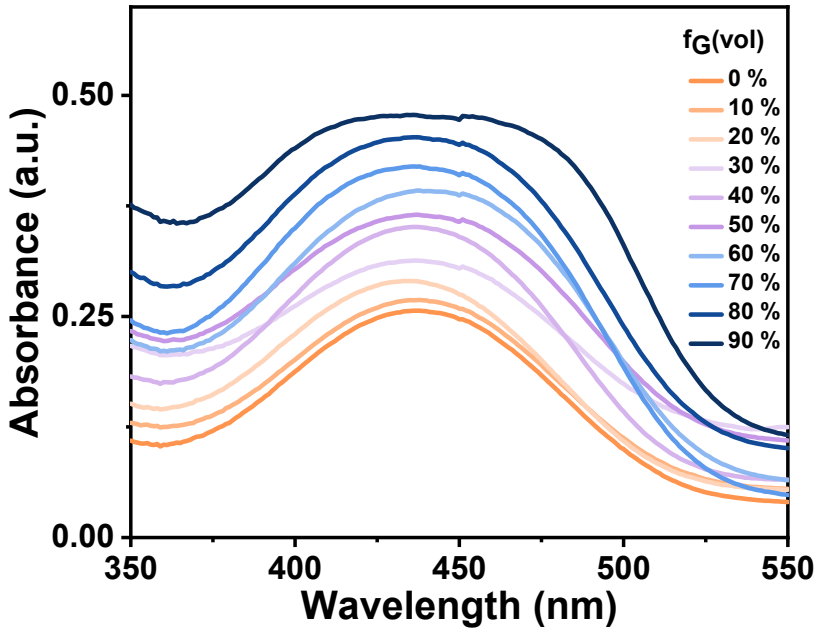


Fig. S6. Absorbance spectra of ZLCN (20 μM) in glycerol/ethanol mixtures with different glycerol fractions (f_G).

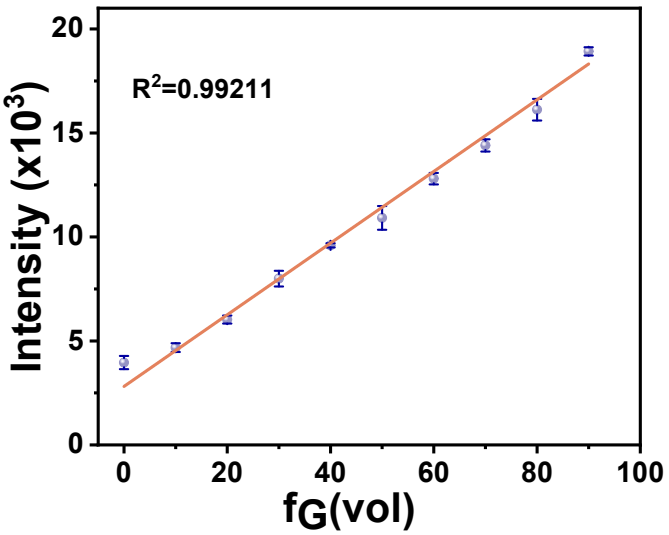


Fig. S7. Linear response between fluorescence intensity of ZLCN and viscosity. λex = 436 nm. λem = 625 nm.

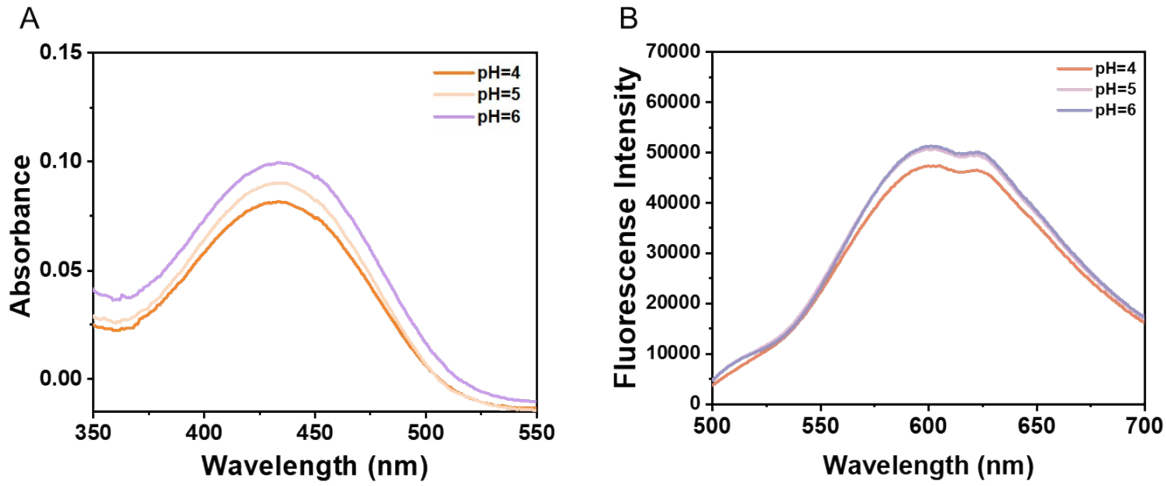


Fig. S8. (A) Absorption and (B) emission spectra of ZLCN recorded using ethanol as a cosolvent in buffer solutions with varying pH values.

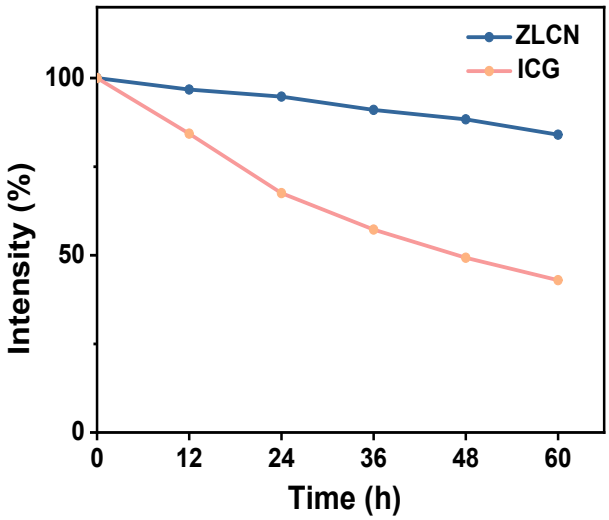


Fig. S9. Stability of ZLCN evaluated in DMEM containing 10% FBS over different incubation

times.

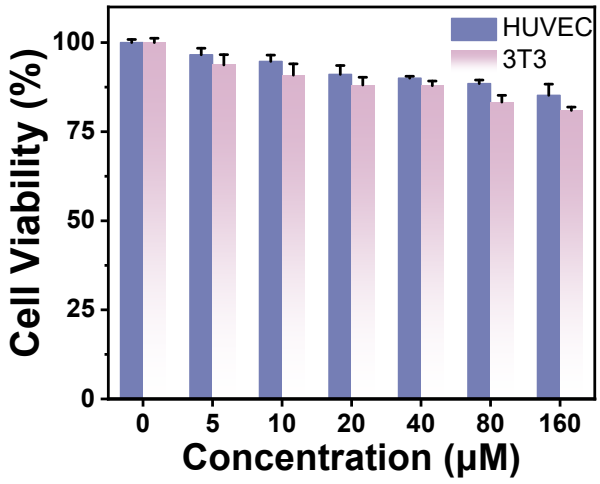


Fig. S10. Cellular toxicity of ZLCN investigated on HUVEC and 3T3 cells.

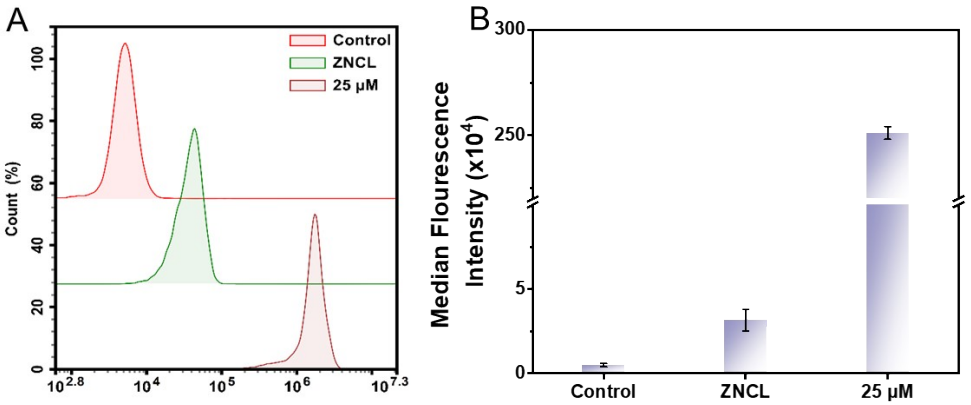


Fig. S11. (A) Flow cytometry analysis of AML12 cells after incubation with ZNCL or ZNCL +Nys (25 μM). (B) Quantification of fluorescence intensity from panel (A).

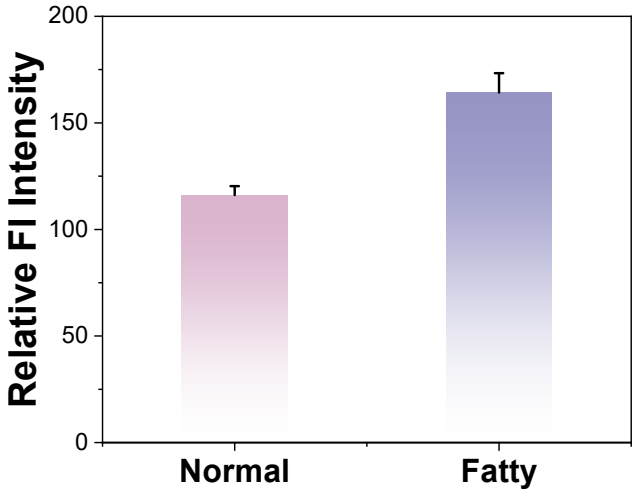


Fig. S12. Relative fluorescent intensity of ZLCN measured from liver tissue in different groups.